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Biosensors for screening kinase inhibitors

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One of the prime fundamentals of biology is the transcription of deoxyribonucleic acid (DNA) into ribonucleic acid (RNA) and translation of RNA into proteins [1]. To regulate various cellular activities, proteins then undergo post-translational modification. Post-translational modification is a chemical modification of the proteins usually caused by covalent bonding with molecules containing phosphate, methyl, acetyl, lipids or carbohydrates. The addition of a phosphate group to a protein is commonly defined as protein phosphorylation. It is catalysed by kinases, which transfer a phosphate group from a phosphate donor to the protein [2]. This modifies the biological activity of the proteins by generating protein dependent functional cell aberrations [3]. It is the proteins with serine, threonine, tyrosine or histidine amino acid residues that undergo this post-translational modification, which brings conformational changes in its structure [4]. Phosphorylation of proteins is a reverse chemical reaction and the opposite of it is called

protein dephosphorylation, which is the removal of phosphate from the protein and is catalysed by the enzymes called protein phosphatases [5].

Phosphate groups play an important part in the various cell mechanisms, acting as building blocks for DNA and RNA. Phosphates are also significant components of the energy generating molecules (e.g. ATP) in all metabolic processes [6]. The role of phosphorylation is significant in the regulation of many processes such as mitosis and carbohydrate metabolism [3] and acts as a switch that turns on and off various cellular mechanisms. Its impact on cellular processes depends on how different proteins behave in their phosphorylated and dephosphorylated states. Phosphorylation was originally discovered by Carl and Gerty Cori in late 1930s as a control mechanism while studying glycogenolysis [1]. However, Fisher and Krebs understood the true nature of glycogenolysis many years later while working on the enzyme phosphorylase kinase, which led to authentic discovery of the protein phosphorylation mechanism. This is because glycogenolysis involved several secondary messengers and other factors, which were discovered between that period. Finally, protein kinase activity was understood when Gene Kennedy in 1954 and Earl Sutherland discovered the secondary messengers which trigger phosphorylation in response to certain cellular stimuli. For instance when the body is in need of energy, phosphorylase kinase becomes phosphorylated switching from its inactive-b to active-a form, which converts the stored glycogen into glucose [7].

Role of Protein Phosphorylation

In this section the role of protein phosphorylation in cellular metabolism is explained with two different examples before explaining how it can then lead to disease. One essential metabolic process which is regulated specifically by protein phosphorylation is

glycogenolysis, i.e. conversion of glucose into energy [7]. In this process, when blood sugar levels are low, the glucagon hormone is released into the blood and binds to the surface of liver cells. This activates the (cyclic adenosine monophosphate (cAMP) that binds to the regulatory receptors of glycogen phosphorylase kinase, turning it on, leading it to become phosphorylated. This phosphorylated state of glycogen phosphorylase kinase leads to phosphorylation of glycogen phosphorylase and activates it to convert glycogen into glucose. The resultant energy is then used by the cells to perform various physiological processes.

There are over 500 protein kinases and about 30% of the human proteins undergo phosphorylation to perform essential cellular activities [8]. Therefore, it is not surprising that abnormal phosphorylation should turn out to be a cause of human disease [9]. There are over 400 diseases that are linked with the phosphorylation of proteins, with most of the diseases caused by mutations in the genes that regulate kinases that alter the phosphorylation sites in a protein. For instance in Alzheimer disease, Tau proteins are the major constituents of intraneuronal and glial fibrillar lesions and are referred to as 'tauopathies'[10], [11]. Molecular analysis has revealed that an abnormal phosphorylation is one of the important events in the process leading to the disorder. Similarly, mutations in somatic cells has been reported to encourage phosphorylation of proteins which induces malignancy in the cells [12]. Figure 1 shows a schematic where phosphorylation of proteins activates the cells and makes it cancerous. It also depicts how an inhibitor can stop the cell from being cancerous.

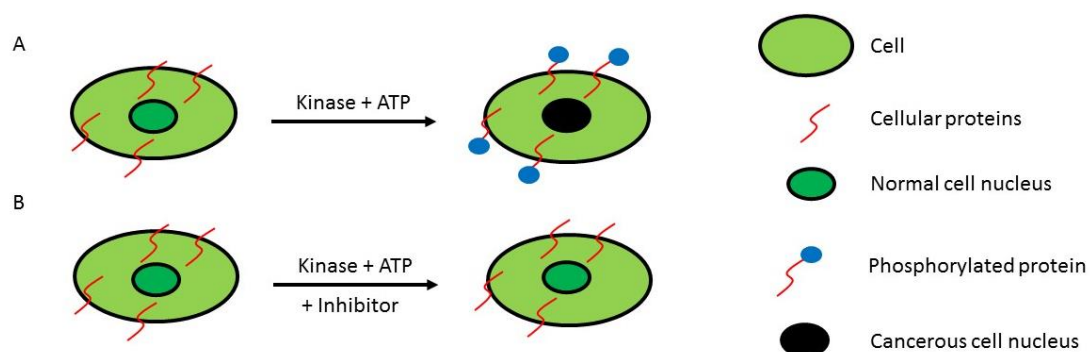


Figure *Error! No text of specified style in document.* schematic of protein phosphorylation A) damage to the cell upon phosphorylation of proteins B) No phosphorylation in the presence of inhibitor

Kinase inhibitors

Kinases inhibitors are the most funded target in the drug discovery market [13]. Kinase inhibitors are a special kind of enzyme inhibitors that slows or impedes the activity of the enzymes called kinases. The most important use of kinase inhibitors is in cancer cure therapy [14]. In this therapy, the patient is injected with a specific kinase inhibitor as a form of chemotherapy. The kinase inhibitors are given as a single therapy or as a combinatorial cancer treatment with radiation therapy [15]. Kinase inhibitors usually work by binding covalently or non-covalently to the kinases [16]. Inhibitors that bind covalently block kinase activity by binding to the Cysteine (Cys) residue either on the ATP active site or around the active site. For instance in epidermal growth factor receptor (EGFR) kinase the Cys residue is on the ATP active site and the extracellular signal-regulated kinase 2 (ERK2) kinase Cys residue is on the d... f... g... -motif (DFG-motif) around the active site. DFG-motif is a combination of three amino acids aspartic acid- phenylalanineglycine (Asp-Phe-Gly) [17]. Non-covalently binding inhibitors bind to the hinge region of the kinase. These can further be classified into ATP-competitive or non-ATP-competitive inhibitors. Competitive inhibitors compete with the high

concentrations of intracellular ATP to attach on the kinase. The non-ATP-competitive inhibitors act by inducing a conformational shift in the kinase such that it is no longer able to function [18].

Characteristics of protein-phosphorylation reaction

The protein phosphorylation reaction involves the release of a proton (H^+) and the transfer of a phosphate group from a donor (e.g. ATP), facilitated by kinase, resulting in a strong negative charge. Measuring the change in the charge of the protein after phosphorylation is one method of identifying protein phosphorylation. Another method could be to detect the release of protons. ATP consists of three phosphoryl groups with the terminal phosphoryl (called as gamma (γ) phosphoryl) group being the one that is transferred to the proteins upon phosphorylation (figure 2) [17], [19]. Therefore, a modification at the γ -phosphoryl group that generates either optical or electrical response can be incorporated. For instance, modifying γ -phosphoryl with a sulphide bond can allow attachment of gold nanoparticles (AuNPs) after transfer to the proteins. Presence of AuNPs can then be analysed using biosensing techniques such as localized surface plasmon resonance technique.

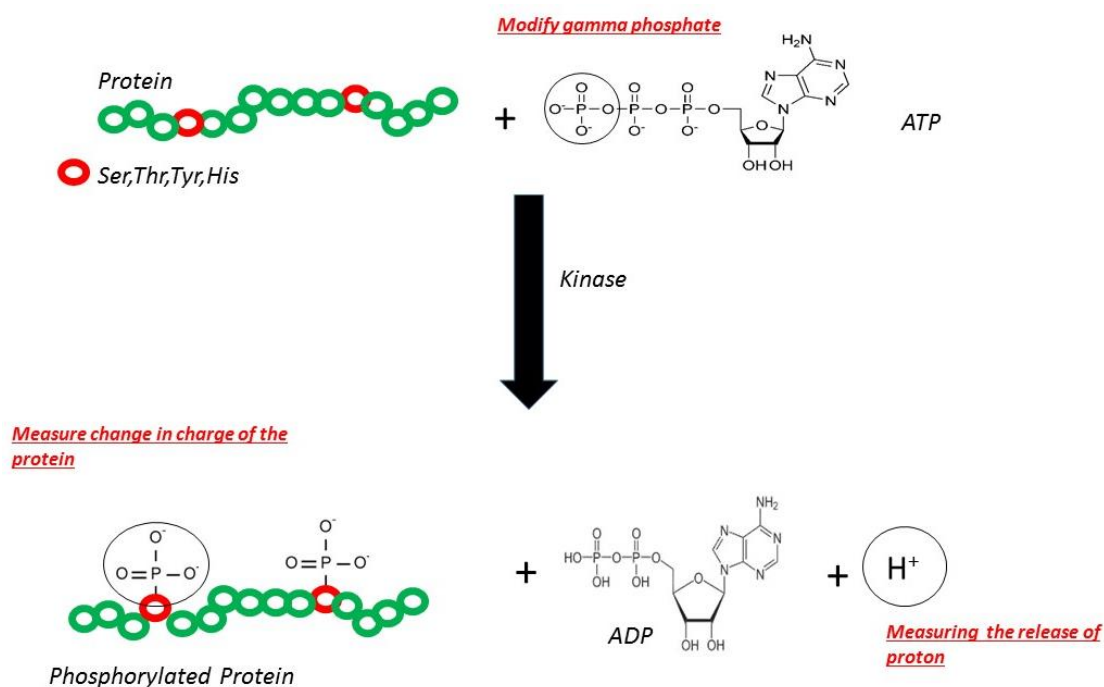


Figure 2: Scheme showing phosphorylation reaction

Methods that have been reported to assess kinase activity include colorimetric, fluorescence, optical, electrochemical, radioactive and spectroscopic techniques. The detection mechanism of most of these usually involves either phospho-specific recognition of proteins or quantifying the phosphorylation product. Recent developments have encouraged the use of nano-scale materials, for example gold and silver nanostructures, quantum dots, graphene oxide, carbon nanotubes, magnetic beads and semiconductor materials to analyse kinase activity. The implementation of these nano hunks has greatly improved the detection limits and selectivity of protein phosphorylation reaction. Yet, there are bottlenecks that have hindered the potential of these advancements to transform into high throughput drug screening tools.

The following sections will aim to present the biography of kinase drug discovery tools developed in academia and industry. In brief it will contextualize the need to develop

technologies for protein phosphorylation analysis by presenting progresses from past to present in the kinase biosensor development.

Conventional Biochemical assay

Conventional biochemical assay uses a label, for instance an antibody, to detect the analyte of interest. Based on the label, analysis tools that are employed to detect the phosphorylation of proteins can be categorised into phospho-specific antibody and radioactive-labelled techniques. These assays are carried out using western blots or on a microtitier plate based ELISA platforms.

Western blot

In pharmaceutical research western blot is a standard method used to assess the post translational modification of proteins. This is because the technique does not require expensive laboratories equipment necessary to perform the experiments and the assay steps are user friendly to carry out. The first step of the assay involves passing the sample through a polyacrylamide gel. The process consists of using sodium dodecyl sulfate (SDS) to impart negative charge on the proteins that assists size dependent movement of proteins through the sample. This whole process of separating protein samples according to their size is called as Polyacrylamide gel electrophoresis (PAGE). Next step involves transfer of the sample from the gel to a membrane usually made of polyvinylidene fluoride (PVDF) or nitrocellulose. From the membrane the analyte of interest is detected using antibodies that produces a chemo-luminescent signal to detect the analyte of interest. Detection of phosphorylated protein and screening of inhibitors of kinase has been demonstrated using this method by in several studies [5]–[13]. While antibody based western blot eliminates the use and waste disposal requirements of radioisotopes, it is a very laborious technique and is not suitable for high throughput

screening of inhibitors. The usual time for the assay varies from 24 to 72 hours so it may take a week to screen for instance 3-5 inhibitors. In addition, this technique provides limited information on the kinetics of the reaction therefore it is only suitable as a validating tool to observe the presence of phosphorylated proteins in an unknown solution [14].

Enzyme-linked immuno sorbent assay (ELISA)

ELISA is another conventional method in pharmaceutical research that is frequently used for measuring protein phosphorylation [15]–[19]. ELISA is usually carried out in a high throughput reaction platform (96 well plate). These ELISA plate are either made up of glass or polystyrene that facilitates immobilisation of enzymes and proteins easily. The technique is more quantitative than western blot because the calibration of the detection mechanisms is standardised. This means that the interpretation of the change in the signal is quantifiable by utilizing a calibrated standard [20]. The detection mechanisms usually involve colorimetric or fluorometric detections [21]–[30]. The analyte of interest for instance the phosphorylated proteins are captured by antibodies that are generally tagged with enzyme which generate fluorescence or colour change signal. There are some loopholes of ELISA that has temporised the use of ELISA plates as trademark drug screening device. One of them is that it doesn't allow detection of the analyte in real time and therefore obtaining information of kinetics and binding specificities of the inhibitor is limited. In addition, the coloured or fluorescent product is prone to quenching effects that may cause the system to give false signals. Lastly, since it involves labelling the phosphorylation event, there is always a possibility of non-specific attachment of the labels that again may provide unreliable signals.

Radioactive labelled assays

The γ -phosphoryl group of ATP, which is transferred to the protein upon phosphorylation, is modified with radioactive isotope ^{32}P , ^{33}P or radioactive orthophosphates [31]. The phosphorylated proteins are then detected using autoradiography. Autoradiography is a technique of measuring the distribution of radioactivity on a surface. The amount of radioactivity is qualitatively proportional to the amount of phosphorylation. Although radioactive assays are quite sensitive in detecting the amount of phosphorylation in a given sample however it has a major disadvantage of not being environmental friendly. Radioactive isotope can damage the cells of the human body and can induce carcinogenic mutations. Therefore carrying out a radioactive assay may cause life-threatening complications. Since radioisotopes has also the potential to damages the proteins, there is a high probability that inhibitor of kinase under test loose its function. Hence, radioactive assays for screening inhibitors of kinases are discouraged to use.

Mass spectroscopy

The addition of phosphate group adds a negative charge to the protein in addition to the increase in its mass. Therefore, it is not surprising that mass spectroscopy (MS) has been regarded as a comprehensive tool in the detection of phosphorylated proteins. In this technique the sample to be analysed is ionized to cations, which are separated according to their mass and charge in a magnetic field. The separated ions are then detected to produce a record of ions as a function of mass/charge. Although MS is a very sensitive technique with high resolution in identifying a single proton, it is not efficient in distinguishing a non-phosphorylated protein from a phosphorylated one. This is mainly attributed to the trade off with the MS principle that comes in when there is a mass and

charge change in protein upon phosphorylation. There is poor ionization of phosphoproteins since they are negatively charged and the ion source that ionises them in positive mode makes them lose their negative charge. This generates a very weak signal from phosphopeptides especially in the presence of high abundance of non-phosphorylated proteins. Secondly, stoichiometric levels of phosphoprotein may be very low and phosphopeptides have relatively low ion abundance in the presence of non-phosphorylated peptides. Lastly, improper sample preparation or peptide fragmentation may cause phosphate groups on phosphoserine and phosphothreonine to decompose. To overcome these drawbacks, several strategies have been proposed to analyse phosphoproteins with MS techniques. This includes antibody enrichment, chemical modification and affinity tag based chromatography methods [32].

Quartz crystal microbalance

Quartz crystal microbalance (QCM) is a sensor that measures mass per unit area as a change in the frequency of an oscillating quartz crystal. The addition of mass upon addition of phosphate group or attachment of functional kinase to its substrate in the presence/absence of a kinase inhibitor can be monitored by QCM to develop drug screening devices. Xu *et al.* [33], [34] described one-step detection strategy for kinases using QCM. An aptameric peptide (IP₂₀) was used to attach PKA kinase via RRNAI motif of the kinase. A change of over -250 Hz was observed on the QCM upon attachment of kinase. These changes were observed in real time and the reaction was completed in 4 minutes. QCM has also been used to distinguish phosphorylated protein from the dephosphorylated ones [35]. In this work authors monitor the attachment of 3 biotinylated phospho-tags on the phosphorylation/dephosphorylated proteins using QCM. The attachment of their most sensitive phospho-tag (monobiotinylated) at a concentration of

0.1 $\mu\text{g/ml}$ revealed a change of around -40 Hz. Other phosphorylation studies using QCM are that of DNA phosphorylation by polynucleotide kinase [36] and the phosphorylation of polycystin-2 to obtain the binding affinities and kinetic constants of the reaction [37]. The advantages of using QCM for kinase assays is that the real time monitoring of the reaction can be achieved in addition to the detection of mass binding events. This may provide useful information of the kinase inhibitor under screening trials. For instance, the kinetics of an inhibitor can provide information on how fast the drug would work or information on its non-specificity could be extracted. However, there are few loopholes in the QCM detection that make researchers a cautious while using QCM.

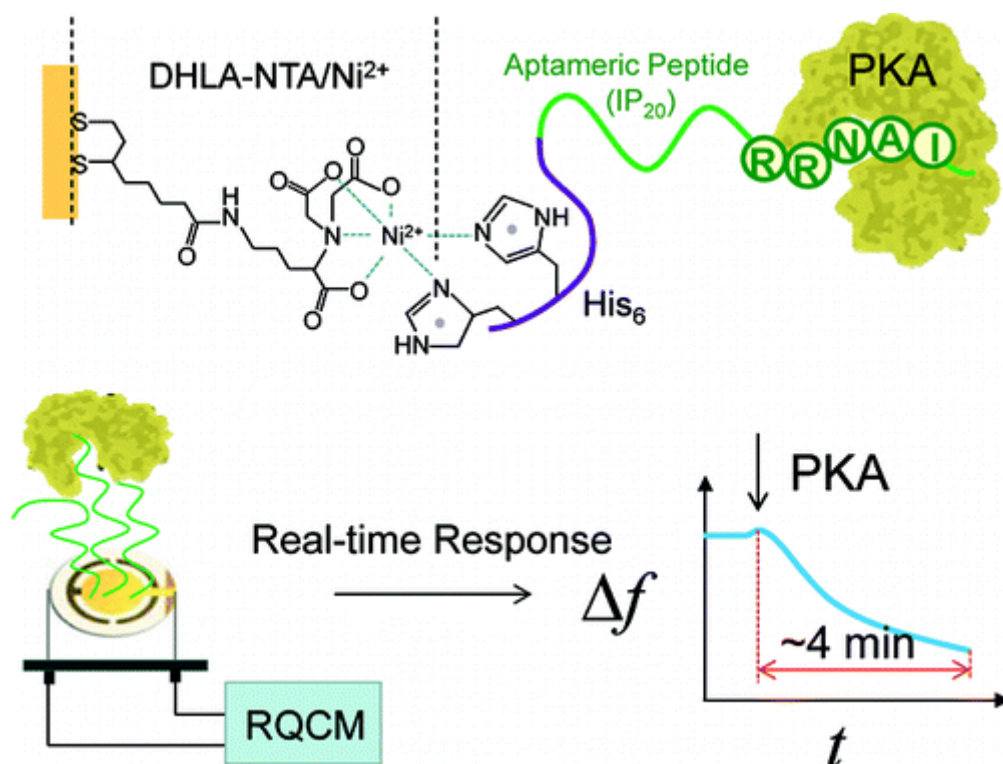


Figure 3 Xu et al. approach to detect kinase using Aptameric sensor on QCM produced from [34] with permission from publisher

The main disadvantages are the over estimation of the mass binding events and an approximate detection limit of 1 ng/cm². Another loophole is that it is difficult to use quartz crystal as a multiple channel platform. In addition, since most of the measurements done on QCM are preferable in a liquid flow, there is a requirement of a steady flow over the sensor surface with at least 50 µl of buffer required [38]. These complexities have limited the use of QCM as an independent validating tool and often a conformation tool is required to support the inferences from QCM.

Nanoparticle based technologies

Nanoparticles have been widely used in biosensing applications either to detect the presence an analyte or enhance the detection signal. For the analysis of kinase assay or the detection of phosphorylated proteins, the use of magnetic particles and the gold nanoparticles (AuNPs) with optical properties has been reported by several research groups [39]. Recently Su et al. induced crosslinking in AuNPs using phosphorylated proteins and quantified it via colorimetric assay [40]. They exploited the negative charge imparted on the proteins upon phosphorylation to aggregate the AuNPs. AuNPs are usually redish pink in colour (the colour becomes lighter when the size of the nanoparticle is increase) and upon aggregation the colour turns blue. Different shades of blue colour can be obtained with different amounts of aggregation. The authors claim that different amounts of phosphorylation generates varied amounts of aggregation. In this way naked eye distinction of phosphorylated proteins from the dephosphorylated can be achieved [41]. Other approaches using AuNPs have also been used to enhance the electrochemical signals from the phosphorylated proteins [42] and employed to either enable optical detection like LSPR [43] or enhance SPR signal [44] [45].

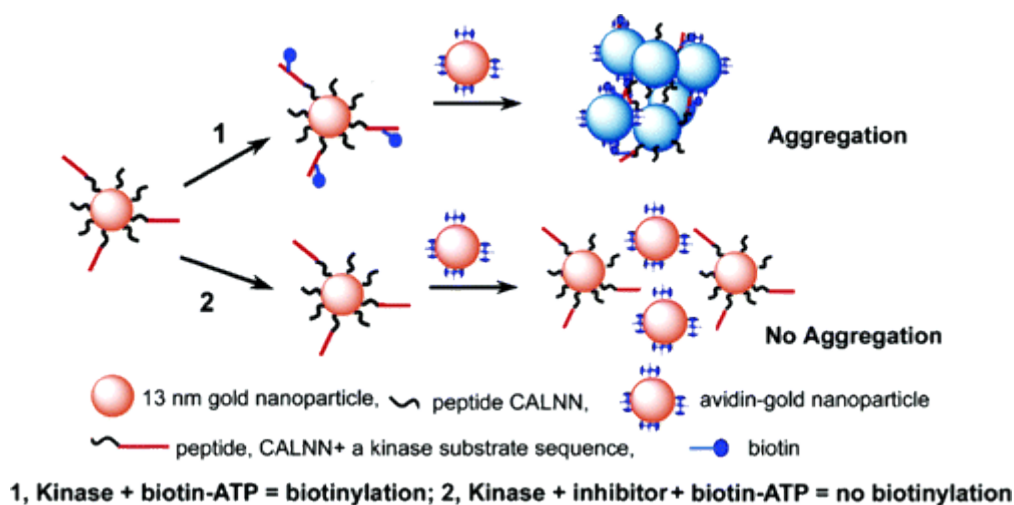


Figure 4 Kinase assay using AuNPs produced from [41] with permission from publisher

Metal oxide nanoparticles have been reported to have specificity for phosphoryl-group and has therefore been used to detect phosphorylation [46], [47]. Chen *et al.* carried out separation of phosphorylated α and β -caseins in a mixed in non-fat milk and egg white in the presence/absence of phosphatase inhibitor using alumina coated magnetic particles[48]. The phosphatase inhibitor avoids the removal of phosphate group from the proteins and allows the phosphorylated protein to attach on the alumina coated magnetic nanoparticles. The magnetic particles were then extracted using magnet and analysed using mass spectroscopy to confirm the attachment of phosphoprotein on them. Similarly Wang *et al.* recently purified phosphorylated peptides using zirconium oxide coated iron-oxide magnetic particles [49]. An approach where phosphorylated peptides were extracted from a solution using zirconium magnetic particles and analysed using a portable glucose sensor was also recently demonstrated by Yang *et al.* [50].

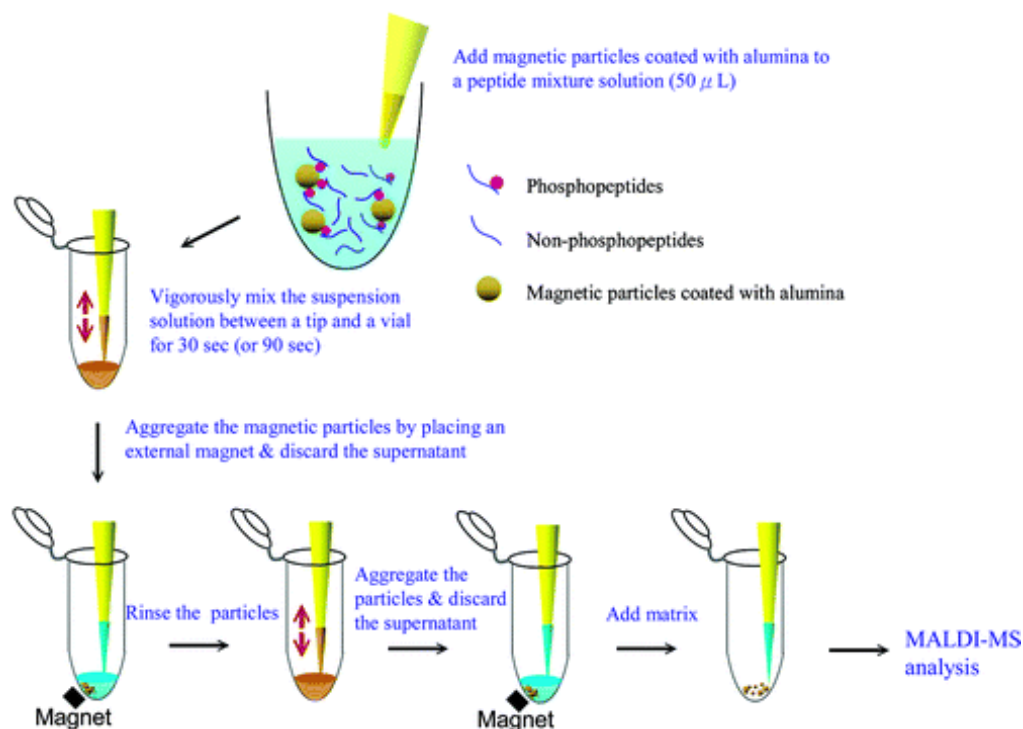


Figure 5 Separation of phosphoproteins using magnetic particles[48]; produced with permission from the publisher

While the detection methodologies using nanoparticles have an advantage of making the system smaller and simple to use, it might have low impact for screening inhibitors of kinase. One reason for this is that inhibitors containing phosphoryl group that have non-specific attachment on the nanoparticles may give a false signal. Secondly it is not easy to produce homogenous nanoparticles of same shape and size therefore the signal may vary from sample to sample. In addition it is not a trivial task to detect real time changes from nanoparticle based detection system. For instance, complex phenomenon like plasmonic coupling or magneto plasmonic coupling occurs when AuNPs or magnetic particles are coupled on real time detection systems like SPR and QCM. This makes the detection of the analytical more qualitative than quantitative.

Electrochemical Biosensors

Electrochemical detection involves commingling of electronics and reaction chemistry. The principle is based on the measurement of change in the surface charge of the electrode due to the activity of analyte in contact with electrode. This change in charge as a function of time is amplified and recorded as current. When a protein gets phosphorylated, it becomes negatively charged and therefore this change in charge makes the electrochemical detection feasible. Electrochemical methods involving potentiometry and amperometry are some of the techniques used to detect protein phosphorylation [51]–[55]. In potentiometry systems the measure of potential difference at zero current detects the analyte. Most commonly used potentiometric biosensor is an ion-sensitive field effect transistor (ISFET) or an open circuit potential (OCP) biosensor.

Formisano *et al.* reported a multimode electrochemical biosensor where OCP was used to detect phosphorylation of proteins in the absence and presence of kinase inhibitor [56]. A real time detection of the kinase that gives an insight of enzymatic kinetics was achieved using this system. In the same work amperometric detection of kinase was achieved using differential pulse voltammetry (DPV). Voltammetry is a type of amperometric technique where the analyte gets oxidized or reduced by the application of a varying voltage between reference and working electrode. The measure of the analyte is given by measuring the changes in the current. In another work Song *et al.* used adenosine-5'-[γ -ferrocene] triphosphate to carry out phosphorylation of proteins, figure 6. In their approach phosphorylated proteins had the presence of ferrocene that was detected using cyclic voltammetry. The corresponding current was the measure of phosphorylation activity [52][57].

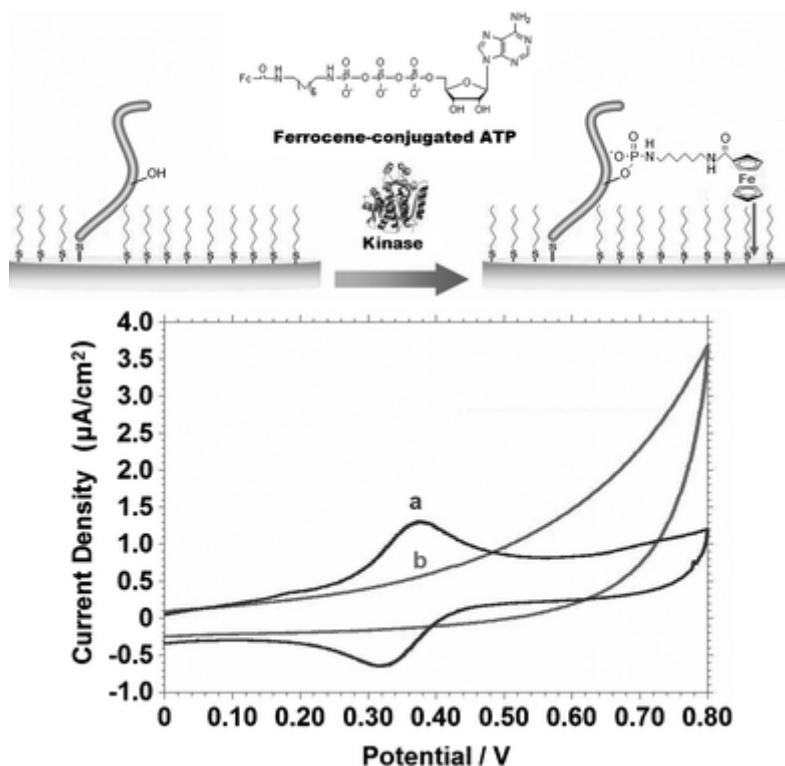


Figure 6 Detection of phosphorylation using cyclic voltammetry: Curve (a) phosphorylation (b) control; [52] produced with permission from publisher

The interface of electrochemistry with nanoparticles has made the electrochemical detection techniques more sensitive and robust. [58]. For instance, Kerman *et al.* demonstrated the application of AuNPs in the detection of protein phosphorylation [51][53]. They immobilized the kinase substrate on screen-printed electrodes and coupled the phosphorylation reaction with biotinylation of it. The biotinylated-phosphorylated kinase binds to the Au nanoparticles coated with streptavidin. In another report they measured differential pulse voltammetry response of AuNPs to monitor the activity of kinase and its substrate [53]. In addition, Li *et al.* proposed a strategy based on HRP

linked catalysis where AuNPs were used for the signal amplification [55]. Impedometric tools like impedance spectroscopy, voltage–current and charge-transfer characteristics have also been reported to be useful in determination of protein phosphorylation events. For instance, change in charge of self-assembled monolayer of peptides upon phosphorylation was also seen by Cao *et al.* Another example with label-free phosphorylation detection was demonstrated by Yitzchaik *et al.* [54]. They immobilized substrate and kinase in monolayers and saw a decrease in the electrochemical impedance of gold electrodes and a negative shift in gate voltage of field effect transistor upon phosphorylation [54].

One of the great advantages of using electrochemical techniques for the phosphorylation detection is its high sensitivity. Secondly the electrodes are simple to use, time saving and large amount of data can be acquired within short duration. In addition, electrochemical detection techniques have the capacity of monitoring binding events that happen during a chemical reactions. However, achieving high throughput using these techniques is extremely challenging that limits its use in drug discovery applications.

MEMS based tools

Micro electro mechanical systems (MEMS) based cantilevers have been very useful in performing label-free biochemical assays. The cantilevers work on the principle of converting mechanical deformation into an electrical signal upon addition of mass. These structures are usually comprised of a transducing element in the form of a capacitive, piezoelectric or piezoresistive actuator integrated to respond to the changes in mechanical structures. Eun Yang's group developed a peptide inhibitor-based cantilever sensor assay for cyclic adenosine monophosphate-dependent protein kinase [59]. They immobilized

the kinase on the bottom surface of the cantilever made up of gold. The addition of phosphate group on the immobilized protein resonated the piezoelectric crystal embedded on the top of the cantilever. Figure 7 shows the scheme of the Yang *et al*'s system.

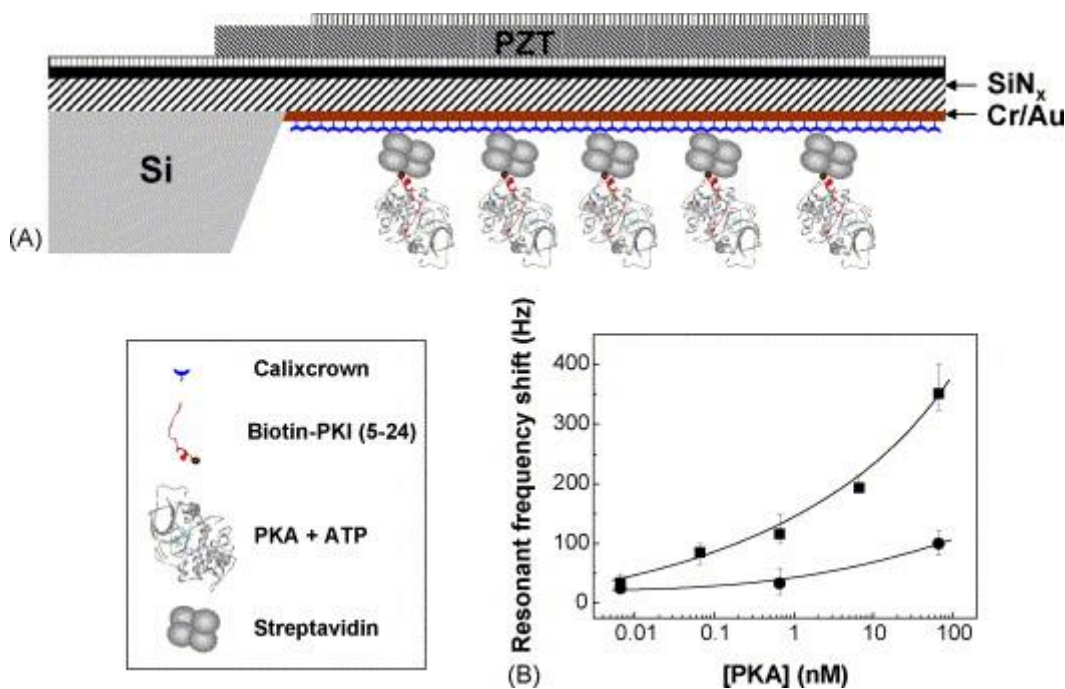


Figure 7 Nanomechanical detection of PKA catalytic subunit on a functionalised cantilever. (A) Schematic representation of the binding of on the Au surface of the PZT cantilever functionalised (B) Results of kinase assay test on cantilever [59]. Produced with permission from publisher.

They exploited the high throughput capabilities of standard MEMS foundry process and produced a device with an array of 12 cantilevers. While MEMS has the ability to produce high throughput drug screening devices, the standard foundry process that enables these processes are immature for biosensing application. For instance, it is challenging to independently expose electrodes in an array to a flow of fluid. The choice of material is also limited to silicon and gold in most processes (for eg. TSMC-

BioMEMS, UMC-MEMS) so it is not trivial to interface nanomaterials to enhance sensitivity of the sensor. Interfacing MEMS with standard complementary metal oxide semiconductor (CMOS) technology and nanostructures presents an exploratory task for researchers to enable development of ultimate drug screening devices.

Atomic Force Microscopy

Since the protein monolayers on a surface get distorted upon phosphorylation so any roughness seen under atomic force microscopic (AFM) would determine the phosphorylation. Snir *et al* [54] reported a change of 9.6 Å in the roughness of the sensor after phosphorylation of peptides. AFM uses a probe on the cantilever structure. Mechanical deflection of the cantilever is the measure of the roughness of the surface. Advantage of using AFM is that it is relatively simple to use. However it is too presumptuous to confirm reaction by just measuring the change in the roughness of sensor as the technique does not provide information on the unwanted or non-specific interactions of the biomolecules on the surface. Therefore it has limited scope in drug discovery applications. Although it may be used as an extra validation tool observe changes on the sensor surface upon phosphorylation.

Infrared Spectroscopy

Infrared spectroscopy is based on the principle that molecules absorb energies at certain frequencies that are characteristic of their structure. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was employed by Goldsztein *et al.* to study phosphorylation of proteins [60]. They found major changes between the wavenumber 1300–1000 cm⁻¹, a region where phosphate vibration occurs. Strongest peak was observed at 1077 cm⁻¹. While an authentic conformation of presence of phosphate can be achieved

using FTIR [61], it is difficult to associate the changes with phosphorylation. This is attributed to the fact that any non-specific attachment of the ATP will show presence of phosphate peaks in the sample and asserting these peaks to phosphorylated protein would be misleading. In addition this technique also requires expensive instrumentation. All these loopholes limit its use for drug screening applications.

Quantum dots

Quantum dots (QDs) are semiconductor based nanoparticles with fluorescence properties. These nanoparticles typically consist of 10 to 100 atoms. A simple assay to detect phosphorylation of proteins was demonstrated by Shiosaki *et al.* using QDs [62]. They demonstrated that QDs interact with the negative charge on peptides upon phosphorylation. The interaction was quantified using fluorescence resonance energy transfer (FRET). FRET is a technique in which there is distance dependent energy transfer between two fluorophores. As shown in figure 8 the peptides were tagged with fluorophores and the negative charge of the phosphorylated peptides increased the distance between the QDs and fluorophore that caused a change in the resonance properties of the system. The authors studied the activity of PKC kinases in the presence and absence of a known inhibitor of PKC. There are few other reports in literature that have explored the properties of QDs to study phosphorylation of proteins [63], [64]. Although FRET provides remarkable sensitivity to detect the phosphorylated proteins and subsequently screen inhibitors of kinase, they are subjected to fluorescence quenching effects. This may occasionally lead to a false signal of detection.

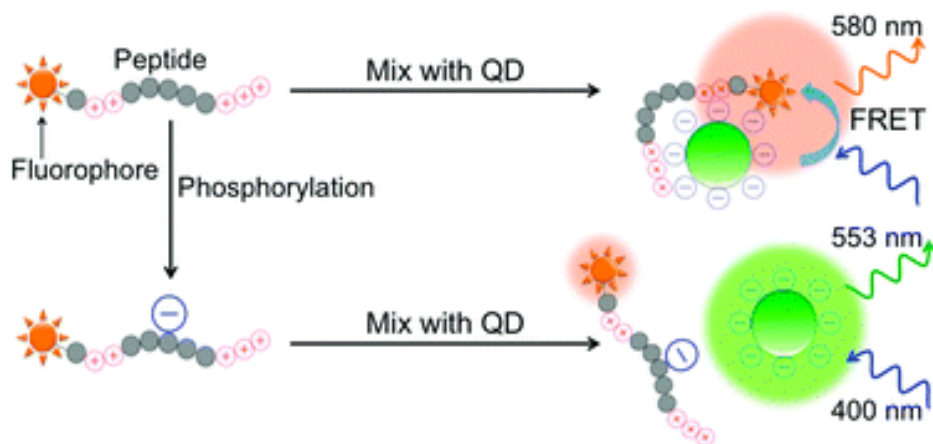


Figure 8 Scheme for using quantum dots for the detection of kinase activity [62]; produced with permission

Photonic crystal

A photonic crystal biosensor was developed by MacConagy *et al.* to obtain dose and time dependent response of phosphorylation of peptides [65] [66]. The device consisted of crystalline colloidal array (CCA) polymerized into a hydrogel matrix. Diffraction measurements from the hydrogel matrix revealed that upon phosphorylation of peptides there is a redshift in the diffraction wavelength of the gel. This is due to the fact that the negative charge of the phosphorylated protein encourages repulsion of crystals in CCA. This was revealed by observation of expansion in the gel upon phosphorylation. Figure 8 shows the expansion of the hydrogen upon activity of kinase.

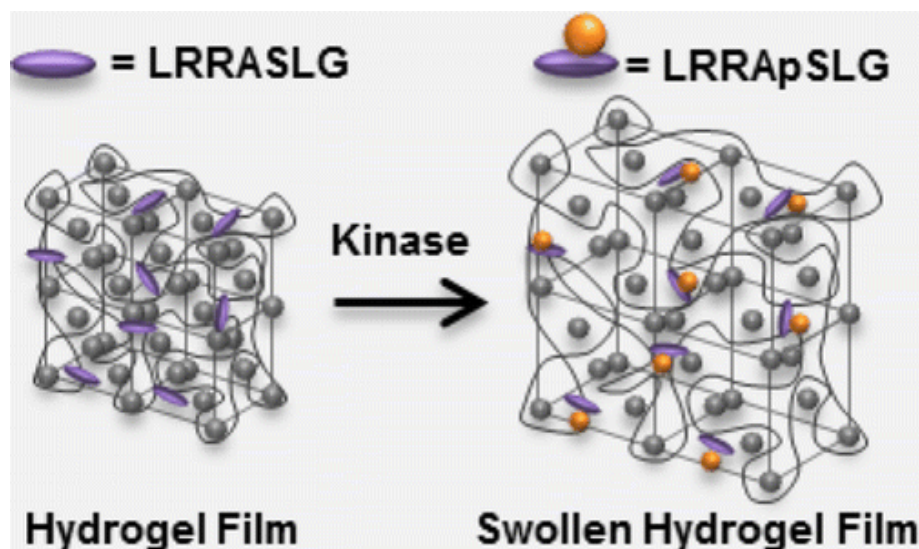


Figure 9 Expansion of hydrogel upon activity of kinase; Obtained from open access article [65]

Contact angle

Contact angle systems measure the wetting properties of the surface. Every surface has a characteristic surface tension that makes it either hydrophobic or hydrophilic in nature. Upon binding of biomolecules to a sensor surface, there are changes in the surface tension of the material that is measured by observing the spread of water on it. The angle that water makes on the sensor surface while it spreads is known as contact angle. Wieckowska *et al* studied phosphorylation of proteins using contact angle [67]. The negative charge of the phosphorylated proteins makes the sensor surface hydrophilic and a reduction of the contact from 67.5° to 36.8° was observed on the gold based surfaces [68]. Usually the measurement system is simple and it requires a camera, optical lens and a source of light to give the right contrast to the droplet. However, the contact angle does not provide any significant information on kinetics of the reaction. Therefore, it is an addition validation tool to confirm changes on the surface of the sensor. Whether the

changes are due to the specific or non-specific interactions of the molecules is asserted by other detection mechanisms.

Field effect devices

Field effect transistor (FET) measures the changes of potential on the surface of an electrode. This research has explored the prospects of using field effect devices as drug screening tool. The potential is usually changed due to change in charge upon a chemical reaction in which case a charge sensitive electrodes, for instance gold electrodes, are used for the measurement of the analyte. The potential change on the FETs may also be due to the change in the pH upon chemical reaction in which case pH sensitive material such as silicon nitride is used as an electrode. Freeman et al. first studied the degree of charging of the gate surface due to kinase activity [69]. They used aluminium oxide gated field effect transistor to follow the phosphorylation of proteins. They carried out simultaneous phosphorylation and dephosphorylation on their sensor and achieved a 40 mV change in the signal upon phosphorylation of proteins. Real time monitoring of the kinetics of the reaction was also followed. Another study that demonstrated phosphorylation/dephosphorylation of peptides using silicon nitride based FETs reported a change of 118 mV [54]. Detection of release of pH associated with phosphorylation of protein, changes in charge upon phosphorylation and demonstrated how kinase inhibitors could be screened was first demonstrated by this research [43], [45], [70].

Major advantage of using FET based devices is its high throughput capabilities. Potential of very large scale integration technology (VLSI) to form an array of FETs to monitor biocatalytic transformation was explored by Ion Torrent on their DNA sequencing chip[71]. Whole genome sequencing was performed on that chip whereby the protons

were produced by adding a nucleotide to each of the several molecules in a clonal colony of DNA [72]. This release of protons was measured by an array of FETs. Similarly if entire human proteome is printed on an array of ISFETs such that there is one type of protein above each FET, then it will be possible to address the whole human proteome and study which protein was phosphorylated with a given kinase [73]. This would also make it possible to know how various inhibitors (drugs) of kinase affect that particular protein phosphorylation [73,74]. Therefore, its application in development of miniaturized rapid drug discovery platform is of prime significance.

Plasmonic biosensors

The quantum associated with collective oscillations of electrons that takes place when light is shinned on a material with specific dielectric constant is known as plasmon. Materials that satisfy the large negative real dielectric constant and small imaginary dielectric constant refractive index show the presence of plasmons and are generally called plasmonic material [75]. The concept of plasmonic biosensor is based on the interaction of surface plasmon wave produced at the interface of the plasmonic material and an isotropic media for light. Plasmonic biosensors can be categorised into surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR) based detection systems.

Thin planar films of gold (< 100 nm) are one of the materials that satisfies the requirements of plasmons. To excite surface plasmons on the gold the most common configuration is the Kretschmann configuration where the light is shinned through a prism at 'just near' to the critical angle of incidence. At angles near to critical angle of incidence, there is total internal reflection of the light and it is allowed to pass through

the other media (gold film). The variations in the light that passes through the other media can be measured as a change in the angle of the total internal reflected light. These variations are usually brought in by the interactions of plasmonic evanescent wave with the biomolecules attach on the surface. On the other hand LSPR works with the similar physics except that the formation of plasmons is on nanoscale materials.

Nordin *et al.* is the only exclusive report that demonstrated the use of SPR biosensors to screen inhibitors of kinase [76]. The work successfully demonstrated the use of SPR to study direct binding of inhibitors to the kinases and a detailed kinetic analyses of their binding events. On the other hand use of LSPR to screen inhibitors of the kinase was first demonstrated by the work reported in the thesis. The LSPR was combined with the field effect devices as complementary biosensors to confirm phosphorylation of proteins [43]. Detailed description of the technique is demonstrated in the following chapters.

2.16 Conclusions

This review reported a survey of techniques used by the researchers to study phosphorylation of proteins for the development of kinase inhibitor screening devices. While highly sophisticated assays and devices have been reported in literature to study kinase activity, the research is still pre-mature to develop a high throughput drug screening device. Therefore, there is a lot of scope to develop simple biosensors to study phosphorylation of proteins. To overcome this there is a need for the researchers from chemistry/biology and other basic sciences to closely work with the researchers in the field of nano/micro-engineering and electronics as biosensors is highly interdisciplinary area of research.

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